

Optimising host cell physiology and stress avoidance for the production of recombinant human tumour necrosis factor α in *Escherichia coli*

Selas Castineiras, Tania; Williams, Steven; Hitchcock, Antony ; Cole, Jeffrey; Smith, Daniel; Overton, Tim

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1 **Optimising host cell physiology and stress avoidance for the production of recombinant**
2 **human tumour necrosis factor α in *Escherichia coli***

3 Tania Selas Castiñeiras^{1,2,3}, Steven G Williams¹, Antony Hitchcock¹, Jeffrey A Cole^{3,4}, Daniel C
4 Smith¹, Tim W Overton^{2,3*}.

5 ¹Cobra Biologics, Stephenson Building, The Science Park, Keele ST5 5SP, UK.

6 ²School of Chemical Engineering, ³Institute of Microbiology & Infection, and ⁴School of
7 Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

8 * To whom correspondence should be sent: t.w.overton@bham.ac.uk +44 (0) 121 414 5306

9 **Keywords:** Heterologous protein; High Cell Density Culture; Fed-batch fermentation; Protein
10 solubility; Biopharmaceutical.

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13 **Abbreviations:** APH, aminoglycoside 3' phosphotransferase; CHO, Chinese Hamster ovary;
14 cGMP, current good manufacturing practices; EMEM, Eagle's minimum essential medium; LB,
15 lysogeny broth; rhTNF α , recombinant human tumour necrosis factor alpha; RPP, recombinant
16 protein production; TCP, total cell protein.

17

18 **ABSTRACT**

19 As high-level recombinant protein production (RPP) exerts a massive stress on the production host,
20 an extensive literature on RPP optimisation focuses on separating the growth phase from RPP
21 production once sufficient biomass has been obtained. The aim of the current investigation was to
22 optimise benefits of the relatively neglected alternative strategy to achieve high level RPP during
23 growth by minimizing stress on the host. High yields of the biopharmaceutical recombinant human

24 Tumour Necrosis Factor alpha (rhTNF α) were obtained by fed-batch fermentation relevant to
25 industrial production based upon parameters that most severely affected RPP in preliminary
26 laboratory scale batch cultures. Decreasing the inducer concentration and growth temperature, but
27 increasing the production period were far more effective for increasing RPP yields than changing the
28 growth phase at which production was induced. High yields of up to 5 g·L⁻¹ of rhTNF α were obtained
29 with minimal plasmid loss even in synthetic media that lack animal-derived components and are
30 therefore fully compliant with regulatory requirements. Most of the product was soluble and
31 biologically active. In summary, stress minimisation was shown to be an effective way to optimise
32 production of rhTNF α . Data generated in shake-flask experiments allowed design of intensified
33 bioreactor cultures in which RPP and growth could be balanced, leading to higher yield of both
34 rhTNF α and biomass than previous fermentations. An additional benefit of this approach is
35 avoidance of lysis during harvesting and downstream processing.

36 INTRODUCTION

37 Recombinant proteins (RP) for use as human biopharmaceuticals represent a commercially
38 important product group for the pharmaceutical industry, with 40 % of pharmaceutical sales
39 accounted for by biopharmaceutical drugs. Seven of the top-selling 10 drug products in 2013 were
40 protein biologics [1]. Bacteria such as *Escherichia coli* are favoured as the production host when the
41 product is relatively simple and does not require glycosylation or other extensive posttranslational
42 modification for function. *E. coli* remains an important host for biopharmaceutical production due to
43 its relative simplicity when compared to mammalian cell systems such as CHO (Chinese Hamster
44 Ovary), and its ease of use [2].

45 An extensive literature documents two major routes for RP production (RPP) in *E. coli*: First,
46 generation of RPs in insoluble inclusion bodies, which although easy to harvest need first to be
47 denatured then refolded *in vitro* to a functional soluble form [3]; Alternatively, generation of soluble
48 functional RP *in vivo* [4]. Although there are industrial examples of both routes, the former pathway
49 relies upon successful refolding following inclusion body denaturation, which for many RPs can be

50 very inefficient, complex and costly. Therefore, production of soluble RP in *E. coli* remains an
51 important objective of process development.

52 Industrially relevant fermentation processes are designed to generate large quantities of RP along
53 with high biomass yields. These conflicting objectives result in severe physiological stress on the
54 bacterial host due to competition between the two processes for metabolic resources such as
55 aminoacylated tRNAs, energy and reducing power. Many successful fermentations avoid this conflict
56 by separating the growth and RPP phases, thereby minimizing the selection of unproductive,
57 plasmid-free bacteria or selection of mutants defective in RP accumulation. In the current study we
58 have exploited a less studied approach involving concomitant growth and RPP under conditions that
59 decrease the stress on the host bacteria [5,6,7]. Previous studies have shown that RPP can be
60 more easily balanced with biomass accumulation by growing bacteria at a lower temperature and
61 inducing production at a lower level by use of weaker promoters or lower inducer concentrations,
62 allowing higher biomass concentrations to be achieved.

63 As RP is generated more slowly, successful folding is enhanced, thus increasing soluble protein
64 production. However, only a very few of the previous studies using this approach have reported the
65 effects of all of the key variables such as the effects of medium composition, temperature, inducer
66 concentration, the structure of the recombinant plasmid and then reported process development into
67 fed batch cultivation. In many cases the target was green fluorescent protein rather than an
68 industrially important product, and the medium components used would not meet current GMP
69 (Good Manufacturing Practice) requirements.

70 Human Tumour Necrosis factor (TNF α) is a cell signalling protein involved in systemic inflammation
71 and its primary role is the regulation of immune cells. TNF α is first synthesised in humans as a 26
72 kDa transmembrane precursor protein, which is proteolytically processed into an active, soluble 17
73 kDa protein that obligately associates into homotrimers [8]. Recombinant human TNF α (rhTNF α) is
74 currently on the market under the international non-proprietary name tasonermin. It is expressed as
75 the soluble 17 kDa monomer in *E. coli*. It was approved by the European Medicines Agency in 1999
76 for the treatment of soft-tissue sarcoma and commercially produced by Boehringer Ingelheim under

77 the trade name of Beromun[®]. TNF α was selected as a model RP in this study due to its commercial
78 relevance, because it has been used for other studies as a model protein for cytoplasmic RPP in *E.*
79 *coli* [9] and since reference material can be commercially obtained. Our first aim was to define
80 parameters that are most significant for the production of soluble rhTNF α in *E. coli* shake-flask
81 cultures. Data from these initial studies were used to direct development of high cell density fed-
82 batch bioreactor cultures that lack components of animal origin. The results demonstrate that stress
83 minimisation can be successfully applied to generate soluble rhTNF α production in an industrially
84 relevant process.

85 **METHODS**

86 *Bacterial strain and plasmids*

87 *E. coli* BL21-T7 (F- *ompT lon hsdS_B(r_B⁻ m_B⁻) gal dcm araBAD::T7RNAP*) sourced from Cobra
88 Biologics (Keele, UK) was used for the production of rhTNF α . The gene coding for hTNF α was
89 synthesised with codon optimisation and cloned into the pLT72 vector (Cobra Biologics, Keele, UK),
90 under the transcriptional control of the T7 promoter. Addition of arabinose to *E. coli* BL21-T7 induces
91 production of the T7 RNA polymerase, inducing expression from pLT72. Additionally, three different
92 vectors were generated containing: (i) the sequence encoding the hTNF α gene and the T7 terminator
93 sequence downstream from the multiple cloning site (pLT72-T7t-TNF α); (ii) the T7 terminator
94 sequence and the kanamycin gene in reverse orientation (pLT72-T7tKan-TNF α); and (iii) the T7 and
95 T2 terminator sequences flanking the kanamycin resistance gene (pLT72-T7tKanT2t-TNF α).
96 Plasmid vectors pLT72 and the pLT72-TNF α were kindly generated by Bruce Humphrey at Cobra
97 Biologics.

98 *Shake-flask growth experiments*

99 In initial experiments, biomass and rhTNF α production in induced and non-induced conditions in
100 shake-flasks were compared using two commonly used media; Lysogeny broth (LB) and Terrific
101 broth (TB). LB agar contained 10 g·L⁻¹ BBL[™] phytone peptone (BD), 5 g·L⁻¹ Bacto[™] yeast extract
102 (BD), 5 g·L⁻¹ NaCl and 15 g·L⁻¹ extra-pure agar (Merck Millipore) in deionised water. LB contained 10

103 g·L⁻¹ BBL™ phytone peptone, 5 g·L⁻¹ Bacto™ yeast extract and 5 g·L⁻¹ NaCl in deionised water.
104 Terrific broth (Life technologies) contained 47 g·L⁻¹ of premade terrific broth powder (equivalent to
105 11.8 g·L⁻¹ SELECT peptone 140, 23.6 g·L⁻¹ yeast extract, 9.4 g·L⁻¹ K₂HPO₄ and 2.2 g·L⁻¹ KH₂PO₄)
106 and 4 mL·L⁻¹ of glycerol in deionised water. Starter cultures were grown overnight at 30 °C and 200
107 rpm from a single colony of bacteria in 10 mL of LB supplemented with 50 µg·mL⁻¹ kanamycin in a
108 20 mL bottle. Cultures were grown in 50 mL of LB or TB supplemented with 50 µg·mL⁻¹ kanamycin
109 in 250 mL baffled shake-flasks. Sufficient inoculum was added to achieve a starting OD₆₀₀ of 0.1.
110 Upon induction, casamino acids were added to cultures to a final concentration of 2 %, as it has
111 been reported that the addition of casamino acids has a beneficial effect on rhTNFα production [10].
112 Fed-batch fermentation methods are described in Supplemental information.

113 For harvest of cell pellets for purification of rhTNFα, the culture was centrifuged at 3,500 g at 4 °C
114 for 30 min (Sorvall RC3B Plus, rotor Sorvall HLR6/H6000A/HBBC). Pellets were resuspended in
115 phosphate buffered saline (PBS; Gibco, Life Technologies) and gently homogenised using a Dounce
116 homogeniser to remove any traces of culture medium. The homogenised cell paste was centrifuged
117 at 7,500 g at 4 °C for 30 min (Sorvall RC53 Plus, rotor Sorvall SS-34). Cell paste was stored at -20
118 °C.

119 *Analysis techniques*

120 The optical density of cultures at 600 nm (OD₆₀₀) was measured using an Amersham Pharmacia
121 Ultrospec 1100 Pro UV/ Visible Spectrophotometer. Culture samples were also serially diluted in
122 PBS and plated onto LB agar for determination of CFU. For plasmid retention analysis, LB agar
123 plates were incubated at 37 °C overnight; colonies were transferred by replica plating to LB agar and
124 LB agar supplemented with 50 µg·mL⁻¹ kanamycin and incubated overnight at 37 °C.

125 *Subcellular fractionation*

126 For separation of soluble and insoluble protein fractions, a volume of culture equivalent to 1 mL at
127 an OD₆₀₀ of 1 was centrifuged at 12,000 g for 10 min. Pellets were re-suspended in 250 µL of 50 mM
128 Tris-HCl pH 8, 10 mM MgCl₂ and 1 µL of benzonase nuclease (Merck Millipore) and incubated on

129 ice. Lysozyme (3 μL of 10 $\text{mg}\cdot\text{mL}^{-1}$; Sigma-Aldrich) was added and samples incubated on ice for 30
130 mins. Cells were lysed using freeze / thaw cycles; a minimum of 3 cycles of freeze (ethanol/dry ice
131 bath) and thaw (37 $^{\circ}\text{C}$) were carried out for each sample. Samples were centrifuged at 12000 g for
132 30 min, to separate the soluble (supernatant) and insoluble (pellet) protein fractions. Pellets were re-
133 suspended in 250 μL of 50 mM Tris-HCl pH 8, 10 mM MgCl_2 , constituting the insoluble protein
134 fraction.

135 *SDS-PAGE*

136 Denaturing SDS-PAGE (using 4-12 % Bis-Tris NuPAGE SDS-PAGE gels and associated buffers
137 (Life Technologies)) and Western blotting (using Nitrocellulose membrane and NuPAGE transfer
138 buffers (Life Technologies)) were performed according to standard methods as detailed in the
139 supplemental information. AlphaEase[®] software (Alpha Innotech) was used to calculate the quantity
140 of rhTNF α as a percentage of total cell protein (TCP). All samples were normalised by OD_{600} before
141 loading on the SDS-PAGE gel, so each lane contained equivalent biomass.

142 *Purification of rhTNF α*

143 The purification of rhTNF α obtained from fermentation studies was carried out by Nicola Barison at
144 Cobra Biologics. A proprietary purification protocol was used for the purification of rhTNF α and only
145 a summary of the process will be described here. The cell paste obtained from fermentation studies
146 was resuspended and cells were disrupted by the use of a high-pressure cell disruption system
147 (Constant systems). The soluble protein fraction was obtained by centrifugation and clarified.
148 rhTNF α was purified by a process comprising an ammonium sulphate precipitation and several
149 chromatography steps including anion exchange and heparin affinity chromatography. The final
150 product presented a purity greater than 95 %, as quantified by densitometry from SDS-PAGE gels
151 (Supplemental Fig. S1).

152 *TNF α cytotoxicity assay*

153 The C3H mouse fibrosarcoma cell line L929, a cell line sensitive to the activity of TNF α , was used
154 to evaluate the activity of rhTNF α produced by fed-batch fermentation [11]. L929 cells were grown

155 in T225 flasks with Eagle's minimum essential medium (EMEM) supplemented with 10 % (v/v) fetal
156 bovine serum (FBS), 2 mM glutamine and 0.1 % NaHCO₃. The cell culture was incubated at 37 °C
157 and 5 % CO₂ for three days. Once they reached confluency, cell cultures were passaged to a new
158 T225 flask by removing the culture medium, washing the cells with PBS, trypsinisation to detach
159 cells, resuspension in fresh EMEM and transfer to new T225 flasks with fresh EMEM.

160 For the cytotoxic bioassay, 6×10⁴ cells suspended in EMEM medium were added to each well of a
161 96 well plate and incubated at 37 °C and 5 % CO₂ for 18 hours. Once confluent, the medium was
162 exchanged for fresh EMEM medium containing 2 µg·mL⁻¹ actinomycin D, a cell growth inhibitor
163 preventing cell proliferation and sensitising the cells to the activity of rhTNFα, leading to apoptosis
164 [11]. Different concentrations of rhTNFα reference material (Life Technologies), rhTNFα purified from
165 cell paste generated in fermentation 1 or buffer were added to the plates and incubated for 12 - 18
166 hours at 37 °C and 5 % CO₂. After incubation, the culture medium was discarded and 200 µL staining
167 solution (0.5 % (v/v) crystal violet in 20 % (v/v) methanol) was added to each well for 10 minutes.
168 The staining solution was discarded by inversion of the plate, and excess staining solution removed
169 by the addition of deionised water. The waste was removed and the L929 cells were solubilised by
170 the addition of 100 µL of 1 % (w/v) sodium dodecyl sulphate solution. The plate was incubated for 1
171 hour on a rotary shaker at 180 rpm. The OD₅₈₀ of each well was measured using a FLUOstar Omega
172 Microplate Reader (BMG LABTECH). The mean absorbance for each triplicate set of standards or
173 samples assayed were calculated. Using the mean absorbance data, the percentage of cytotoxicity
174 was calculated using equation 2:

$$175 \quad \% \text{ cytotoxicity} = \left[1 - \frac{\text{absorbance of sample}}{\text{absorbance of zero TNF}\alpha \text{ control}} \right] \times 100 \quad (2)$$

176 The concentration of purified rhTNFα giving rise to a cytotoxicity value of 50 % (LD₅₀) was
177 determined.

178 **RESULTS AND DISCUSSION**

179 *Selection of culture medium for the production of rhTNFα*

180 Expression of rhTNF α was driven from an arabinose-induced T7 expression system, similar to the
181 widely-used DE3 / pET system [12]. *E. coli* strain BL21-T7 was used as a host, carrying a
182 chromosomal T7 RNA polymerase gene under the control of an arabinose-inducible promoter. The
183 gene encoding rhTNF α was cloned into pLT72 (Cobra Biologics) under the control of a T7-dependent
184 promoter. In initial experiments, *E. coli* BL21-T7 transformed with either pLT72-TNF α or the empty
185 vector (pLT72) were grown with aeration in Lysogeny broth (LB) or Terrific Broth (TB). At an OD₆₀₀
186 of 1, half of the cultures were induced with a final concentration of 0.2 % (w/v) arabinose and
187 casamino acids were added [10]. Biomass accumulation, culturability (colony forming units), plasmid
188 retention and protein production were analysed (Figs. 1 & 2). Growth of cultures transformed with
189 either the control plasmid or the production plasmid stopped soon after induction. This was expected
190 because production of T7 RNA polymerase, even without production of an RP, induces stress
191 responses in *E. coli* [13]. The final biomass concentration in non-induced cultures in LB was higher
192 than in TB. For each medium, non-induced cultures containing the empty vector and the vector
193 encoding rhTNF α grew similarly. However, for induced cultures, TB cultures grew faster and reached
194 a higher OD₆₀₀ than LB cultures.

195 SDS-PAGE analysis (protocol in supplementary materials) revealed that rhTNF α accumulated
196 gradually after induction, reaching a maximum of 20 % of the total cell protein after 24 hours of
197 growth for both TB and LB (Fig. 2). Very little rhTNF α was present in cells before induction or in non-
198 induced cells after 24 hours growth, revealing that this expression system offers tight regulation of
199 RPP. Bacterial pellets harvested after 24 hours growth were also fractionated into soluble and
200 insoluble fractions. SDS-PAGE revealed that around 55 % of the rhTNF α was present in the soluble
201 fraction for both media. As a result of its buffering capacity and the slight improvements noted in
202 growth and culturability, TB was selected for use in further experiments.

203 SDS-PAGE analysis identified an unexpected protein band (~31 kDa) in induced cultures of cells
204 carrying both the empty vector and the vector encoding rhTNF α . The concentration of this unknown
205 protein band accounted for up to 10 % of the total cell protein (in samples from induced cultures
206 carrying the empty vector). It was hypothesised that this unknown protein could be the product of
207 the kanamycin resistance gene, aminoglycoside 3' phosphotransferase (APH), which has a

208 comparable molecular weight. The lack of terminator sequences downstream of the multiple cloning
209 site on the backbone of vector pLT72 may have allowed read-through by the T7 RNA polymerase,
210 leading to the overproduction of APH.

211 *Effect of inducer concentration and induction point on rhTNF α production*

212 Addition of 0.2 % (w/v) arabinose as an inducer in the previous experiment resulted in stress as
213 evidenced by growth arrest, a decrease in viability and plasmid loss (potentially due to a decrease
214 in culturability of plasmid-containing, productive, bacteria), even in cultures containing the empty
215 vector. The effect of different inducer concentrations (1 %, 0.2 %, 0.05 %, 0.02 % or 0.002 %
216 arabinose, added at an OD₆₀₀ of 1) was tested. Bacteria transformed with the empty vector (pLT72)
217 or the vector coding for rhTNF α (pLT72-TNF α) under non-inducing conditions were used as controls
218 (Fig. 3).

219 Growth of cultures induced with 1 % to 0.02 % arabinose was arrested following arabinose addition
220 and more than 80% of the bacteria were plasmid deficient after 24 h. Furthermore, the final biomass
221 concentration after 24 h was inversely proportional to the inducer concentration used for cultures
222 induced with a concentration of arabinose ranging from 1% to 0.02 %. In contrast, growth of cultures
223 induced with 0.002 % arabinose was only slightly inhibited (Fig. 3a), showing a similar growth profile
224 as the non-induced cultures or cultures containing empty vector, and more than 80 % of these
225 bacteria had retained the plasmid after 24 h (Fig. 3b).

226 The concentration of rhTNF α in cells after 24 hours growth was similar in all cultures (Fig. 3c),
227 independent of the arabinose concentration used, showing that the lowest concentration of
228 arabinose, 0.002 %, was sufficient to fully induce the T7 expression system.

229 The effect of changing the point of induction in shake-flasks was evaluated by inducing RP by adding
230 0.2% arabinose at an OD₆₀₀ of 0.5, 2 or 3 (Supplemental Fig. S2). Unlike changing the inducer
231 concentration, there were no large differences between cultures induced at different cell densities.
232 Cultures induced at an OD₆₀₀ of 0.5 grew more slowly after induction but reached higher cell densities
233 than those cultures induced at an OD₆₀₀ of 2 or 3 (Supplemental Fig. S2a). rhTNF α productivity levels

234 were similar for cultures induced at an OD₆₀₀ of 0.5, 2 or 3 (Supplementary Fig. S2b). However, the
235 final OD₆₀₀ of cultures induced at an OD₆₀₀ of 0.5 was higher than in cultures induced at an OD₆₀₀ of
236 2 or 3, which resulted in a higher final rhTNFα yield.

237 *Effect of temperature on rhTNFα productivity*

238 As well as lowering inducer concentration, stress minimisation can be achieved by decreasing the
239 temperature of growth, thus slowing growth and protein production rates. This has been previously
240 been shown to favour accumulation of recombinant proteins in a soluble form [6,14]. Cultures were
241 grown as before in TB supplemented with casamino acids, but at temperatures of 37 °C, 30 °C, or
242 25 °C. Expression of rhTNFα was induced by the addition of arabinose to a final concentration of 0.2
243 % at OD₆₀₀ = 1, cell samples were harvested 4 hours after induction, separated into soluble and
244 insoluble fractions and analysed by SDS-PAGE (Fig. 4a). The proportion of rhTNFα in the soluble
245 fraction increased as the growth temperature decreased.

246 To investigate the interplay between inducer concentration and temperature of growth, further
247 cultures were grown as before at 25 °C and at OD₆₀₀ = 0.5 induced with concentrations of arabinose
248 between 0.2 % and 0.001 %. Plasmids were retained for 24 h by uninduced cultures or cultures
249 transformed with the empty vector (Fig. 4b). Although >90 % of bacteria induced with 0.2 %
250 arabinose had lost the plasmid within 24 h, plasmids had been retained by most of the bacteria at
251 much higher induced concentrations at 25 °C than at 30 °C (Compare Figs. 3b and 4b). Production
252 of rhTNFα production was fully induced with 0.005 % and 0.002 % arabinose, but was induced less
253 with 0.001 % arabinose (Fig. 4c).

254 *Intensification of rhTNFα production in fed-batch fermentations*

255 The optimal conditions for the production of rhTNFα defined during shake-flask studies were
256 transferred to 5 L fed-batch fermentations using medium A, a semi-defined culture medium
257 formulation obtained from Cobra Biologics (as described in supplemental information),
258 supplemented with 2 % casamino acids. Production of rhTNFα was induced with arabinose to a final
259 concentration of 0.005 % at an OD₆₀₀ of 0.5 after 3 hours of growth (Fig. 5). The culture grew after

260 inoculation reaching a final OD₆₀₀ of 90.8 after 48 hours (Fig. 5a). Although μ initially exceeded 0.4
261 during the initial batch phase of growth, it decreased below 0.4 after 6 hours. The CFU dropped after
262 7 hours growth, and plasmid retention dropped below 90 % after 11 hours (Fig. 5b). After 48 hours,
263 only 2 % of the bacteria had retained the plasmid. Although rhTNF α had accumulated to 22 % of
264 cellular protein after 26 h, it did not significantly increase after that point (Fig. 5c). The increase in
265 biomass between 26 h and 48 h did not correlate with an increase in rhTNF α productivity, as
266 expected since the plasmid retention of the culture was low and decreasing. Production of the 31
267 kDa APH protein had also accumulated by 9 h post-induction. Analysis of soluble and insoluble
268 cellular fractions revealed that the majority of rhTNF α was present in the soluble fraction (Fig. 5d).

269 Taken together, these data revealed that the fermentation conditions as defined in shake-flask
270 studies were a good starting point for fermentation development, as rhTNF α was generated in the
271 soluble fraction in significant quantities and induction of rhTNF α production did not cause growth
272 arrest or immediate decreases in CFU or plasmid retention. However, harvesting the culture after 30
273 h when the percentage of cellular protein that was rhTNF α was the greatest (25 %) would not have
274 resulted in high overall rhTNF α yield due to the low biomass (OD₆₀₀ < 40). This indicates that
275 allocation of resources to growth and RPP was unbalanced. In addition, production of APH from
276 pLT72-TNF α could have increased the metabolic burden on the cells, or provided a pool of APH to
277 daughter cells sufficient to confer kanamycin resistance even in the absence of plasmid.

278 *Improvement of vector design for minimisation of APH synthesis*

279 Although high yields of rhTNF α were obtained in bacteria transformed with plasmid pLT72-TNF α ,
280 plasmid deficient bacteria were detected 24 h post-induction under all growth conditions tested.
281 Attempts were therefore made to decrease the stress on the host further by decreasing expression
282 of APH, which was suspected to be the abundant 31 kDa protein detected by SDS PAGE. Three
283 modified plasmids were constructed (Fig. 6a), and plasmid retention and rhTNF α accumulation
284 during growth in small scale batch cultures were compared with those of the original plasmid. For
285 these experiments, bacteria were grown at 30 °C in Terrific Broth and RPP was induced with 0.02

286 % arabinose. These conditions were known from previous experiments to show high plasmid loss,
287 so any improvement in plasmid retention would indicate reduced stress [6].

288 In contrast to the 20 % plasmid retention by pLT72-TNF α transformant, 40 % of the bacteria
289 transformed with plasmid pLT72-T7t-TNF α in which the commonly-used T7 terminator was cloned
290 downstream of the TNF α gene had retained the plasmid 24 h post-induction (Fig. 6b). Similar results
291 were obtained with plasmid pLT72-T7tKanT2t-TNF α with both the T7 and the T2 terminators after
292 the TNF α gene and the kanamycin gene in reverse orientation. The T2 terminator was chosen to
293 prevent recombination that could have occurred if a second copy of the T7 terminator was inserted
294 into the plasmid. However, plasmid retention was further improved (~80 %) in cultures carrying the
295 vector with the T7 terminator sequence and the kanamycin resistance gene in the opposite
296 orientation to the rhTNF α gene (pLT72-T7tKan-TNF α). SDS-PAGE analysis of whole cell proteins at
297 different time points during growth revealed no significant differences between the four vectors in
298 terms of production of rhTNF α (Fig. 6c). The abundance of the 31 kDa protein was slightly lower
299 with the vector containing the T7 terminator sequence (pLT72-T7t-TNF α); and decreased further for
300 vectors with the sequence coding for kanamycin resistance gene in reverse orientation (pLT72-
301 T7tKan-TNF α and pLT72-T7tKanT2-TNF α). These data confirm the identity of the 31 kDa protein as
302 APH. Plasmid retention data identified pLT72-T7tKan-TNF α as the optimal construct due to its low
303 rate of plasmid loss, indicative of lowered stress.

304 *Improvement of fed-batch fermentation using the improved expression vector*

305 The fed-batch fermentation was repeated with pLT72-T7tKan-TNF α , containing the T7 terminator
306 sequence and the gene coding for the kanamycin resistance gene in reverse orientation
307 (Supplemental Fig. S3). As before, the culture grew continuously, reaching a final OD₆₀₀ of 68 after
308 48 h (Supplemental Fig. S3a), although the OD₆₀₀ only increased slightly after 33 h. The specific
309 growth rate during the batch phase was comparable to the previous fermentation. CFU values
310 decreased slightly less following induction (Supplemental Fig. S3b) compared to the fermentation
311 using pLT72-TNF α (Fig. 5a), whereas plasmid retention remained at close to 100 % throughout.

312 The quantity of rhTNF α was comparable to the fermentation using pLT72-TNF α , with 25 % of cellular
313 protein being rhTNF α after 27 h (Supplemental Fig. S3c). After 48 h, the majority (>80 %) of rhTNF α
314 was in the soluble fraction. The use of the improved vector design helped to minimise plasmid loss
315 during fermentation, avoiding the overgrowth of plasmid-free cells, and therefore, non-productive
316 cells at the last stages of the fermentation process. However, this did not significantly increase the
317 quantity of rhTNF α generated.

318 *Removal of casamino acids from fed-batch growth medium*

319 Good Manufacturing Practices (cGMP) for pharmaceutical products recommend that animal-derived
320 products should be excluded from bacterial culture medium to eliminate risks from zoonotic viruses
321 and transmissible spongiform encephalopathies. To develop a GMP compliant fermentation process,
322 casamino acids (a complex medium component derived from casein) were removed from medium A
323 and replaced with 14 g·L⁻¹ of ammonium sulphate and 0.3 g·L⁻¹ of calcium chloride. Bacteria
324 transformed with plasmid pLT72-T7tKan-TNF α were induced at an OD₆₀₀ of 0.5 with 0.005 %
325 arabinose and sampled for up to 32 h post-induction. The biomass concentration increased steadily
326 reaching a final OD₆₀₀ of 72 (Supplemental Fig. S4a). However, no great increase of the cell biomass
327 was observed after 32 h, and in contrast to previous fermentations the growth rate decreased
328 immediately following rhTNF α induction, (Fig. 5 & Supplemental Fig. S3). There was no decrease in
329 CFU following induction of RPP (Supplemental Fig. S4b), but in contrast to 100 % retention of the
330 plasmid in the presence of casamino acids, plasmid-free bacteria started to overgrow the culture
331 after 9 h growth (Supplemental Fig. S3). The concentration of rhTNF α increased after induction,
332 reaching a maximum of 26 % of total cell protein after 24 h growth (Supplemental Fig. S4c). The
333 concentration of rhTNF α decreased after 24 h, presumably due to outgrowth of plasmid-free, non-
334 productive cells. The concentration of rhTNF α in the soluble fraction was lower than in previous
335 fermentations, with only 50 % of the rhTNF α in the soluble fraction after 48 h. Thus removal of
336 casamino acids was detrimental to the fermentation process. Ben-Bassat *et al.* [15] and Hoffmann
337 *et al.* [11] reported that casamino acids increased recombinant protein yields and/or stability,
338 presumably due to enhanced supply of amino acids allowing more rapid protein synthesis without
339 the need for generation of amino acids *de novo*. Casamino acids contains more free amino acids

340 and more peptide fragments of smaller molecular mass (<250 Da) than the yeast extract also present
341 in the medium [16].

342 *Fermentation with an alternative medium composition*

343 The semi-defined medium C (composition in supplementary information) is fully GMP compliant and
344 has been used successfully both by pharmaceutical companies and to produce model proteins in *E.*
345 *coli* fed-batch fermentations [7,9,17]. This medium was therefore used in 5 L fed batch fermentations
346 to generate rhTNF α from bacteria transformed with plasmid pLT72-T7tKan-TNF α . As before, the
347 addition of the feed was started 10 hours after inoculation and the specific growth rate (μ) during the
348 fed batch phase was maintained at 0.1 h⁻¹ by the use of an exponential feeding profile. The culture
349 was induced at an OD₆₀₀ of 0.5 by the addition of arabinose to a final concentration of 0.005 %. The
350 pH was maintained at 7.0 by the addition of 20 % NH₄OH or 5 M HCl.

351 Exponential growth in medium C was faster than in media A or B, peaking at $\mu = 0.815$ h⁻¹ (Fig. 7a).
352 Unlike previous fermentations, the growth rate increased following induction of RPP, then decreased.
353 CFU measurements consistently increased throughout the fermentation (Fig. 7b) and plasmid
354 retention remained at around 100 % throughout. The concentration of rhTNF α increased gradually
355 after induction, reaching a maximum of 30 % of the total cell protein between 24 and 30 h of growth
356 (Fig. 7c). At the end of the fermentation, rhTNF α constituted 25 % of the total cell protein, being
357 primarily accumulated in the soluble fraction, with less than 30 % of rhTNF α in the insoluble fraction.
358 Volumetric rhTNF α productivities of the four fermentations are compared in Supplemental Table S1.
359 Overall, this fermentation process successfully generated a greater yield of rhTNF α than previous
360 fermentations, the majority being accumulated in a soluble form, minimising plasmid loss and using
361 a culture medium compliant with cGMP guidelines.

362 *Bioassay of rhTNF α activity*

363 The final goal of any fermentation process is the production of a soluble and active product, and
364 protein solubility is often a good indication of activity. However, this statement is not always true, and
365 recombinant proteins might accumulate in a soluble form but with an incorrect conformation, which

limits or abolishes biological activity. Therefore, the activity of rhTNF α was measured using a bioassay [18]. rhTNF α was purified from the cell paste generated in fermentation 1 (Fig. 5; purified rhTNF α shown in Supplemental Fig. S1). The C3H mouse fibrosarcoma cell line L929, which is sensitive to hTNF α , was used to evaluate the activity of the purified rhTNF α . The basis of the bioassay is that unlike dead cells killed by hTNF α , live L929 cells are stained by the dye crystal violet (CV).

The activity of the purified rhTNF α was calculated as the percentage of cytotoxicity by comparing the amount of dye taken up by cells incubated with different quantities of rhTNF α . rhTNF α samples purified from fermentation 1 had a 50 % cytotoxicity (LD₅₀) value at a concentration of 0.349 ± 0.035 ng·mL⁻¹, comparable to the LD₅₀ of 0.265 ng·mL⁻¹ stated by the manufacturer for this batch of rhTNF α . This is within the concentration range of 0.05 to 20 ng·mL⁻¹ in which for most *in vitro* applications hTNF α exerts its biological activity. Overall, the result of the cytotoxicity assay showed that the optimization of the fermentation conditions have led to the successful production of soluble and active product and the rhTNF α produced during by fed-batch fermentation was active and stable.

Conclusion

In summary, stress minimisation has been demonstrated to be an effective tool for the optimisation of the production of the human biopharmaceutical rhTNF α . Data generated in shake-flask experiments allowed design of intensified bioreactor cultures in which RPP and growth could be balanced, leading to high quantities of both rhTNF α and biomass. Balanced growth, allowing RP to accumulate along with biomass and thus maintaining cell health and viability, is important not only for biomass and RP accumulation during the fermentation, but also for harvest. Unhealthy or stressed bacteria are more difficult to harvest by centrifugation and subsequent downstream processing steps [5].

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398

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446 **FIGURE LEGENDS**

447 **Figure 1. Selection of culture medium for the optimisation of the production of rhTNF α .** *E. coli*
 448 BL21-T7 carrying the empty vector (pLT72) or the vector encoding rhTNF α (pLT72-TNF α) were
 449 grown at 30 °C in LB (a,c,e) or TB (b,d,f); half of cultures were induced with 0.2 % arabinose at an
 450 OD₆₀₀ \approx 1. Samples were taken at regular intervals and (a,b) OD₆₀₀, (c,d) CFU and (e,f) plasmid
 451 retention determined. Data shown are single values for CFU and plasmid retention and mean values
 452 from replica flasks for OD₆₀₀, error bars are \pm 1 standard deviation.

453 **Figure 2. Accumulation of rhTNF α in cultures grown in LB or TB culture media.** *E. coli* BL21-
 454 T7 carrying the empty vector (pLT72) or the vector encoding rhTNF α (pLT72-TNF α) were grown in
 455 (a) LB or (b) TB at 30 °C; half of cultures were induced with 0.2 % arabinose at an OD₆₀₀ \approx 1 and
 456 samples were taken at regular intervals. Whole cell lysates were separated by SDS-PAGE and
 457 protein stained with colloidal blue. M, marker; BI, before induction. The quantity of rhTNF α is
 458 expressed as a percentage of whole cell protein at the bottom of the gel. Samples collected at 24
 459 hours were also fractionated to obtain soluble (24S) and insoluble (24I) cell fractions facilitated by
 460 the addition of lysozyme (shown on right). The \sim 31kDa protein presumed to be APH is shown on the
 461 right.

462 **Figure 3. Effect of the inducer concentration on the production of rhTNF α .** (a) Growth of *E. coli*
463 BL21-T7 carrying the empty vector (pLT72, non-induced) or pLT72-TNF α , incubated at 30 °C and
464 induced with between 1 % and 0.002 % arabinose at OD₆₀₀ \approx 1. (b) Plasmid retention after 24 hours
465 of growth as in (a). (c) SDS-PAGE showing accumulation of rhTNF α from whole cell lysates obtained
466 24 hours post-inoculation. Data shown are mean values from two replica flasks for OD₆₀₀ and plasmid
467 retention, error bars are \pm 1 standard deviation.

468 **Figure 4. Effect of the temperature and inducer concentration on the production of rhTNF α .**
469 (a) SDS-PAGE gel showing the accumulation of the rhTNF α in the soluble (Sol) and insoluble (Ins)
470 fractions 4 hours after induction. Cultures were induced with 0.2 % arabinose and grown at 37 °C,
471 30 °C or 25 °C; samples were fractionated and rhTNF α quantified by densitometry to give the
472 percentage of rhTNF α in the soluble and insoluble fractions. (b) Plasmid retention of cultures carrying
473 either the empty vector (pLT72) or pLT72-TNF α were grown at 25 °C under non-induced conditions
474 or induced at OD₆₀₀ \approx 0.5 with 0.2 % to 0.001 % arabinose. Data shown are mean values from two
475 replica flasks, error bars are \pm 1 standard deviation. (c) SDS-PAGE gel showing the accumulation of
476 rhTNF α from whole cell lysates after 24 hours of growth as in (b).

477 **Figure 5. Production of rhTNF α by fed-batch fermentation.** *E. coli* BL21-T7 pLT72-TNF α was
478 grown at 25 °C in medium A and induced with 0.005 % arabinose at OD₆₀₀ \approx 0.5 (t = 3h; solid arrow).
479 Feed was started at t = 10h (dashed arrow). (a) Growth as determined using OD₆₀₀ and specific
480 growth rate. (b) CFU and plasmid retention. (c) SDS-PAGE analysis of whole cell lysates. The 3h
481 sample is immediately before induction. rhTNF α and APH are indicated, along with densitometric
482 analysis of the percentage of cellular protein that is rhTNF α . The “-ve” sample is *E. coli* BL21-T7
483 pLT72 (empty vector) after 24 hours of growth (non-induced). (d) Samples were separated into
484 soluble and insoluble fractions. rhTNF α and lysozyme are indicated.

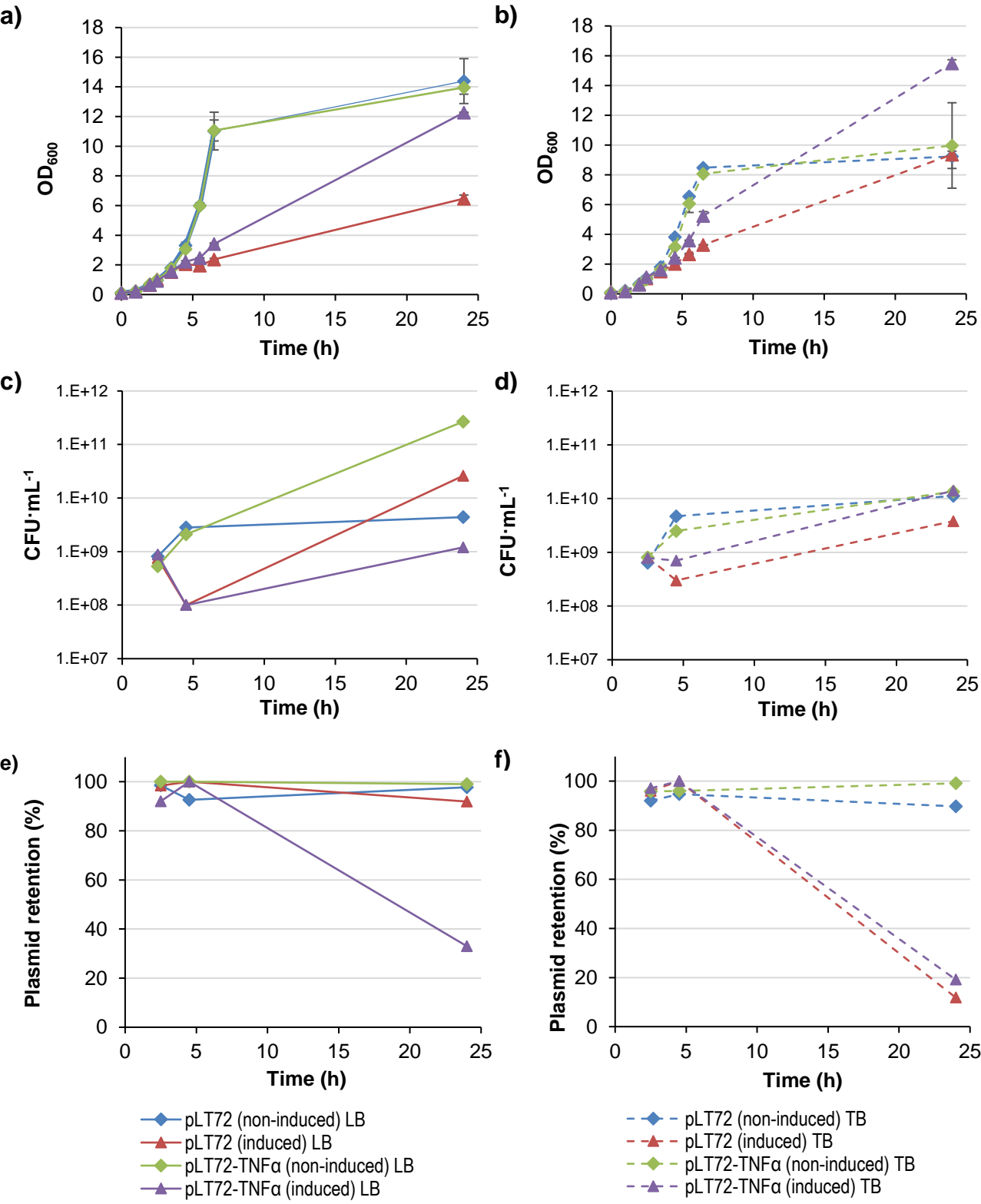
485 **Figure 6. Optimisation of vector design to confirm the identity of the 31 kDa protein as APH**
486 **and to minimise its production.** (a) Schematic of the new vector designs. Arrows indicate genes,
487 stem-loops terminators (T7t and T2t). (b) Plasmid retention after 24 hours growth of cultures carrying
488 the empty vector (pLT72) or the 4 vectors as in (a) grown at 30 °C under non-inducer or induced

489 (0.02 % arabinose at $OD_{600} \approx 0.5$) conditions. Data shown are mean values from two replica flasks,
490 error bars are ± 1 standard deviation. (c) SDS-PAGE gel showing the accumulation of rhTNF α and
491 APH in whole cell lysates after 4 h, 8 h, 10 h, 12 h and 24 h growth as in (b).

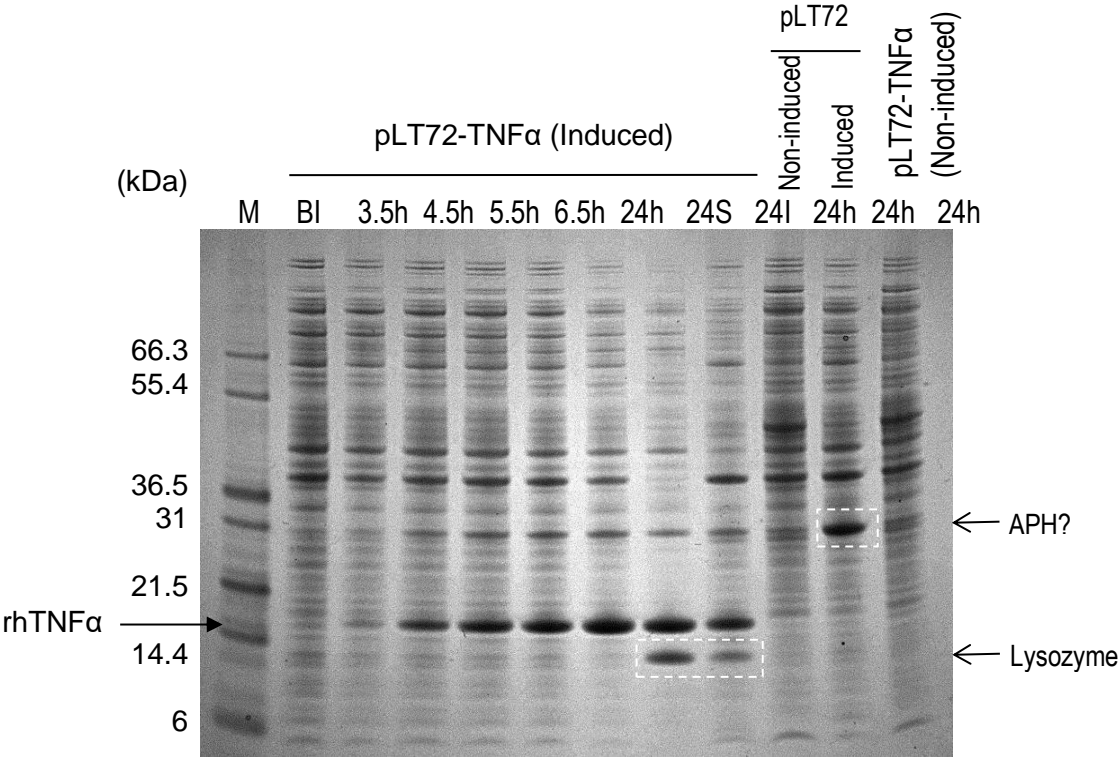
492 **Figure 7. Production of rhTNF α using an alternative fed-batch medium composition.** *E. coli*
493 BL21-T7 pLT72-T7tKan-TNF α was grown at 25 °C in medium C and induced with 0.005 % arabinose
494 at an $OD_{600} \approx 0.5$ (t = 5h; solid arrow). Feeding was started at t = 10h (dashed arrow). (a) Growth as
495 determined using OD_{600} and specific growth rate. (b) CFU and plasmid retention. (c) SDS-PAGE
496 analysis of whole cell lysates. The 5h sample is immediately before induction. rhTNF α is indicated,
497 along with densitometric analysis of the percentage of cellular protein that is rhTNF α . The “-ve”
498 sample is *E. coli* BL21-T7 pLT72 (empty vector, non-induced) after 24 hours of growth. Samples
499 obtained at t = 48h were also fractionated into soluble (Sol) and insoluble (Ins) fractions to determine
500 the quantities of soluble and insoluble rhTNF α .

501

502 **FIGURE 1**

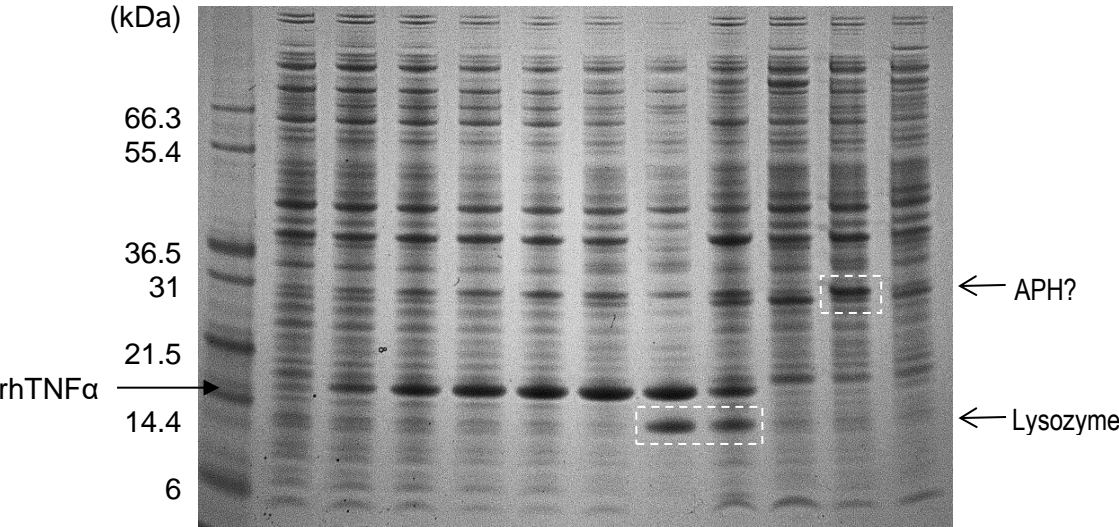


a)



% cellular protein that is rhTNFα: 5% 10% 13% 15% 20%

b)



% cellular protein that is rhTNFα: 5% 11% 12% 15% 20%

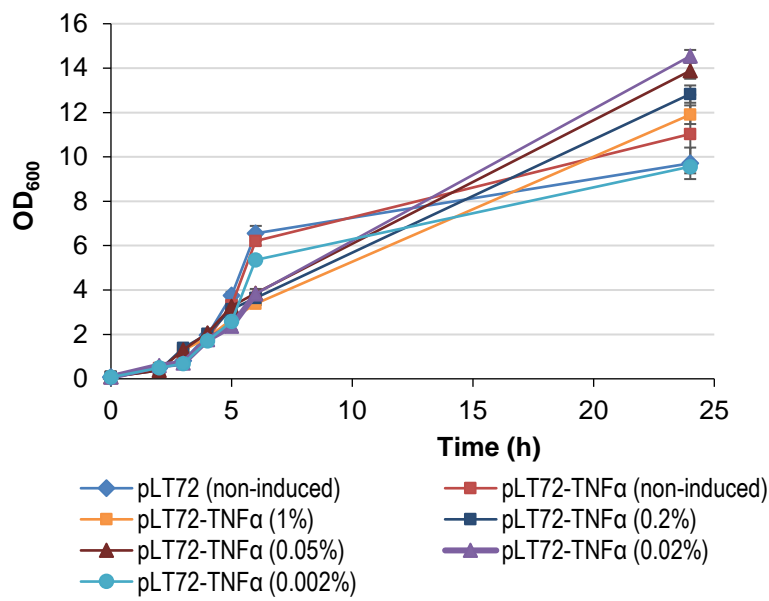
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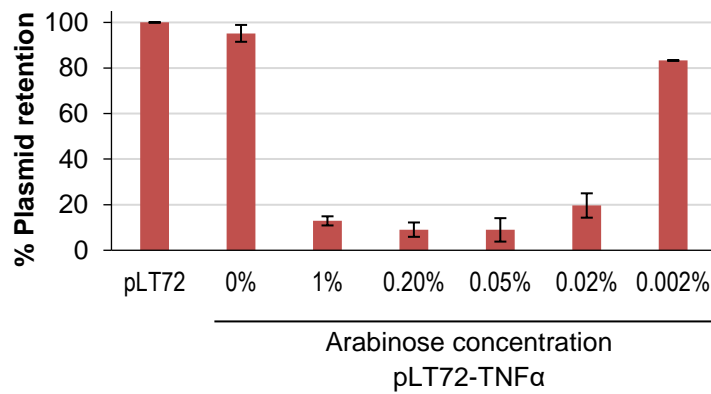
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511 **FIGURE 3**

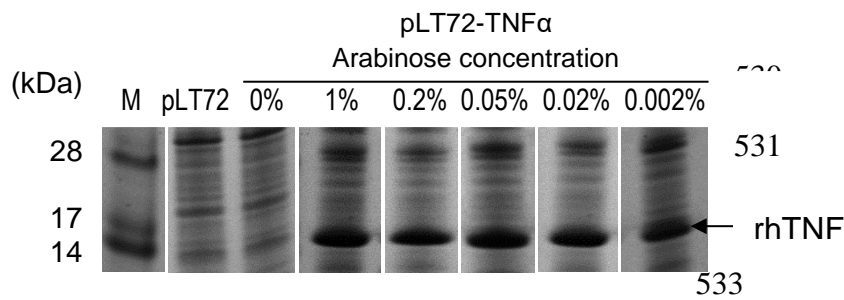
512 **a)**



522 **b)**



529 **c)**



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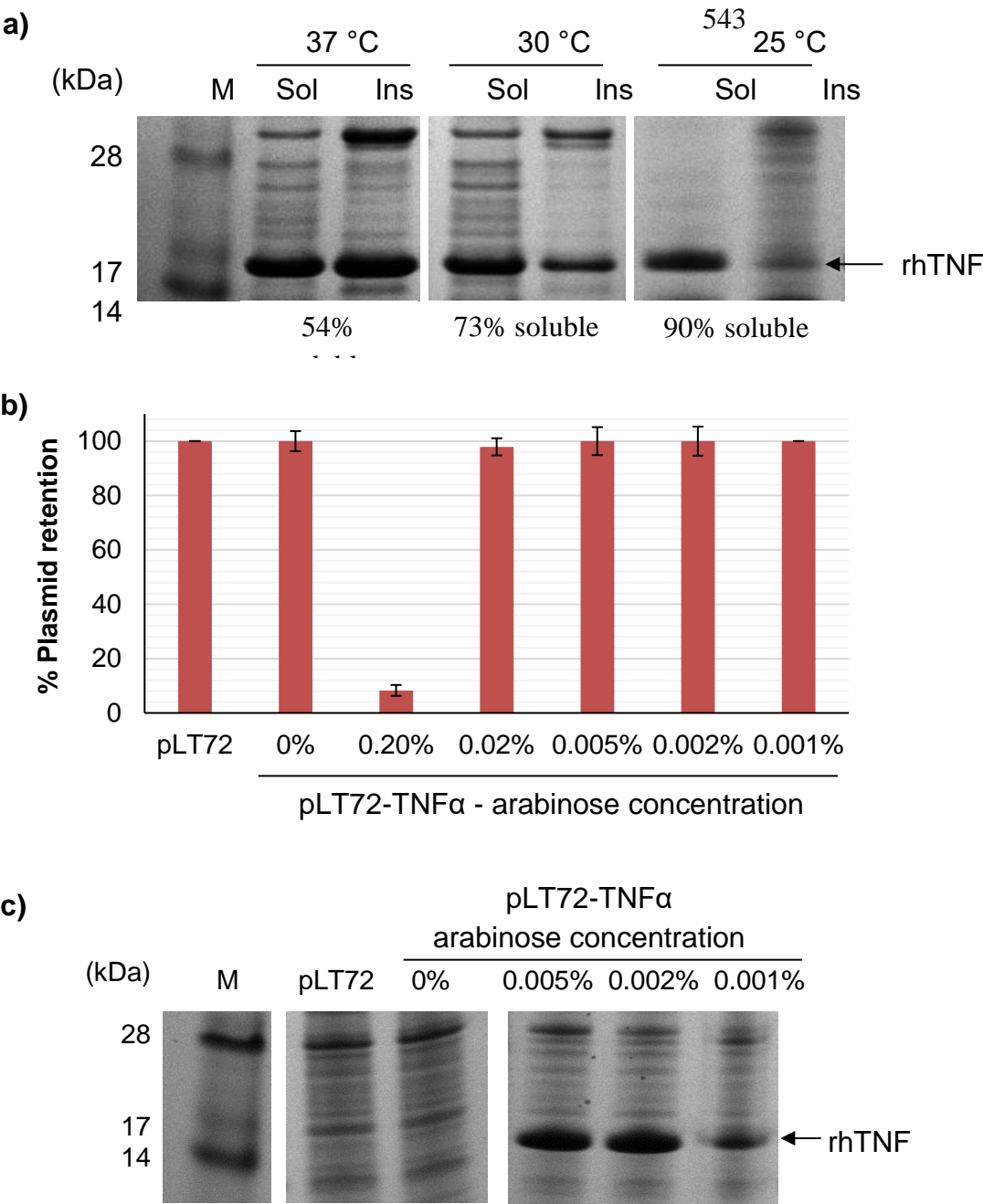
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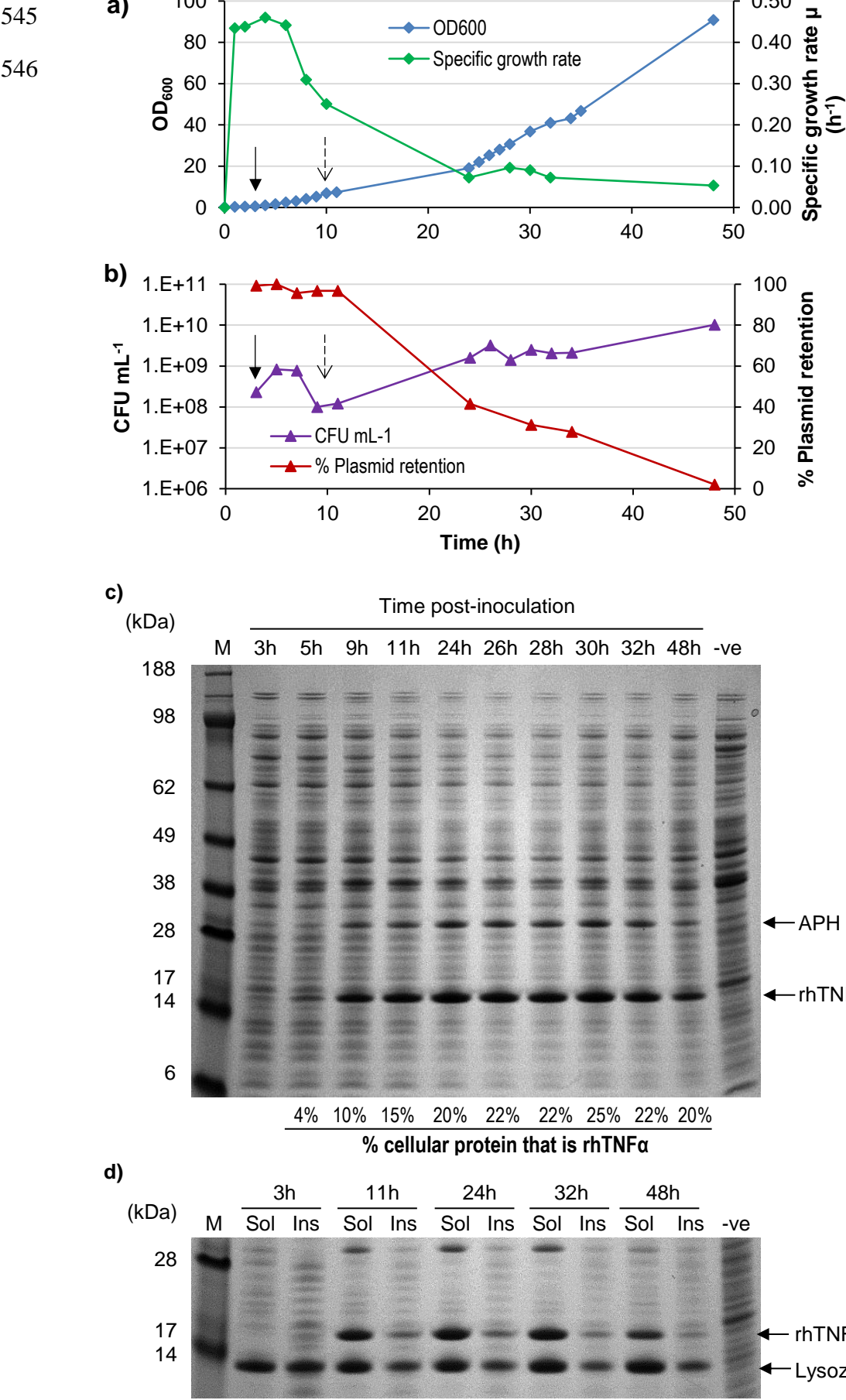
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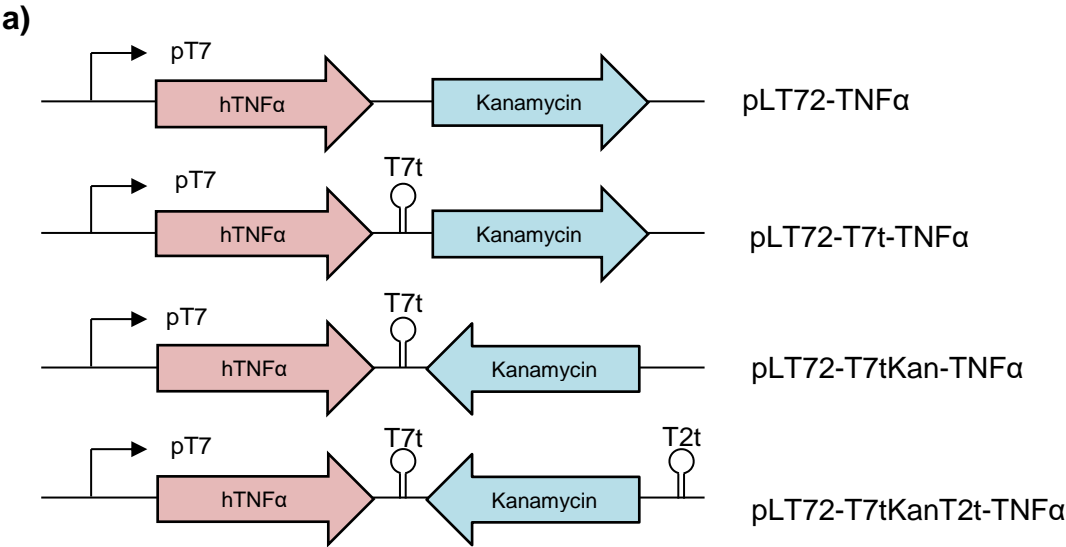


544 FIGURE 5



547 **FIGURE 6**

548



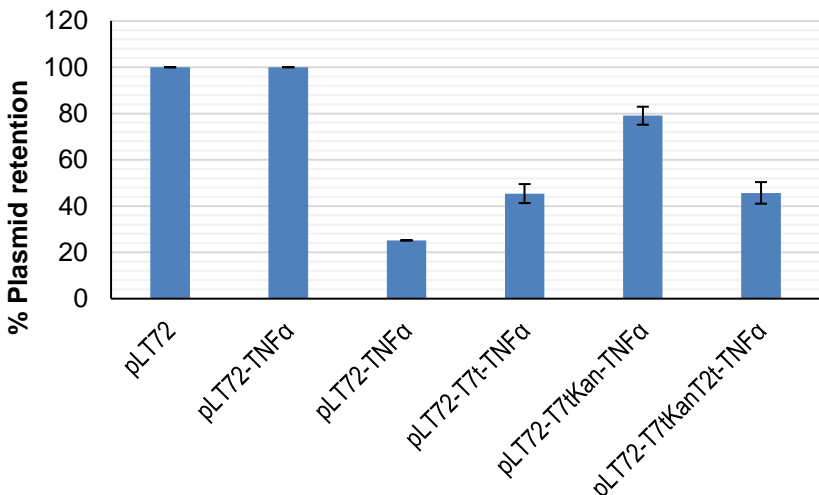
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550 **b)**

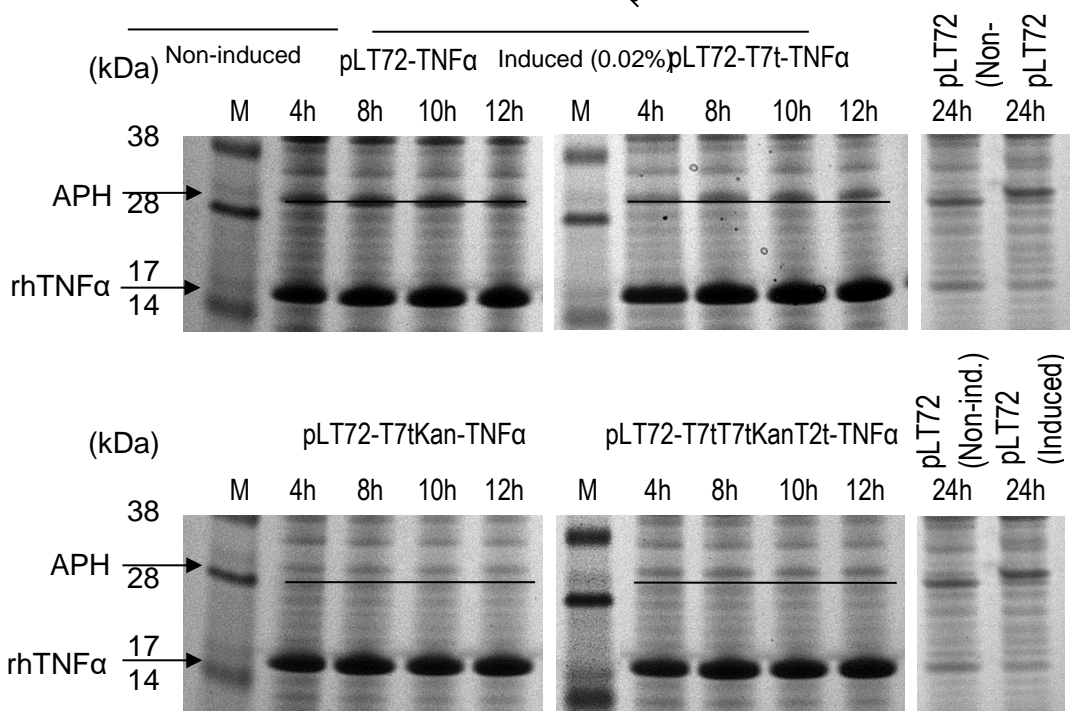
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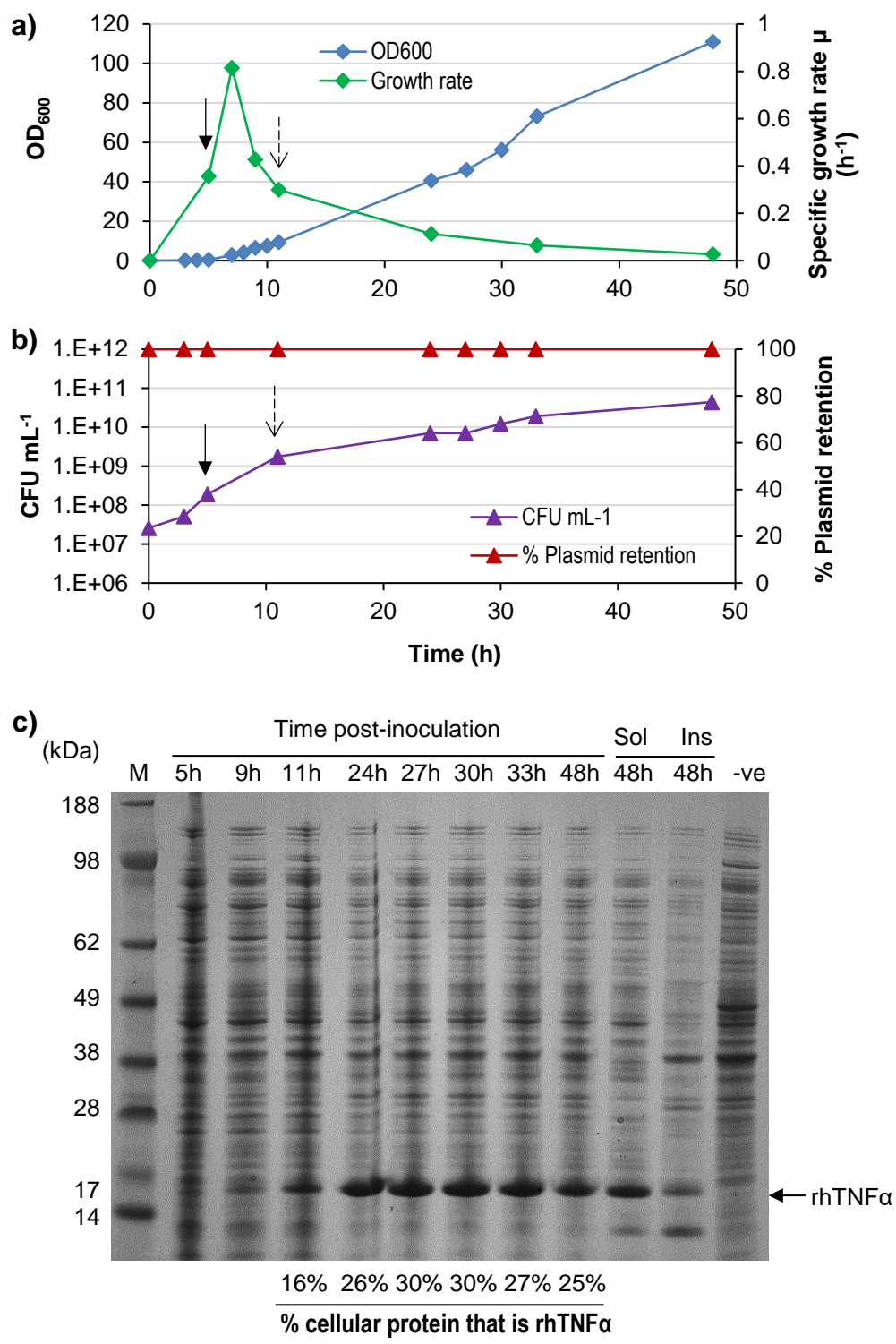
c)



554 FIGURE 7

555

556



557 **Supplemental information for:**

558 **Optimising host cell physiology and stress avoidance for the production of**

559 **recombinant human tumour necrosis factor α in *Escherichia coli***

560 T Selas Castiñeiras^{1,2,3}, SG Williams¹, A Hitchcock¹, JA Cole^{3,4}, DC Smith¹, TW

561 Overton^{2,3*}.

562 ¹Cobra Biologics, Stephenson Building, The Science Park, Keele ST5 5SP, UK.

563 ²School of Chemical Engineering, ³Institute of Microbiology & Infection, and ⁴School of

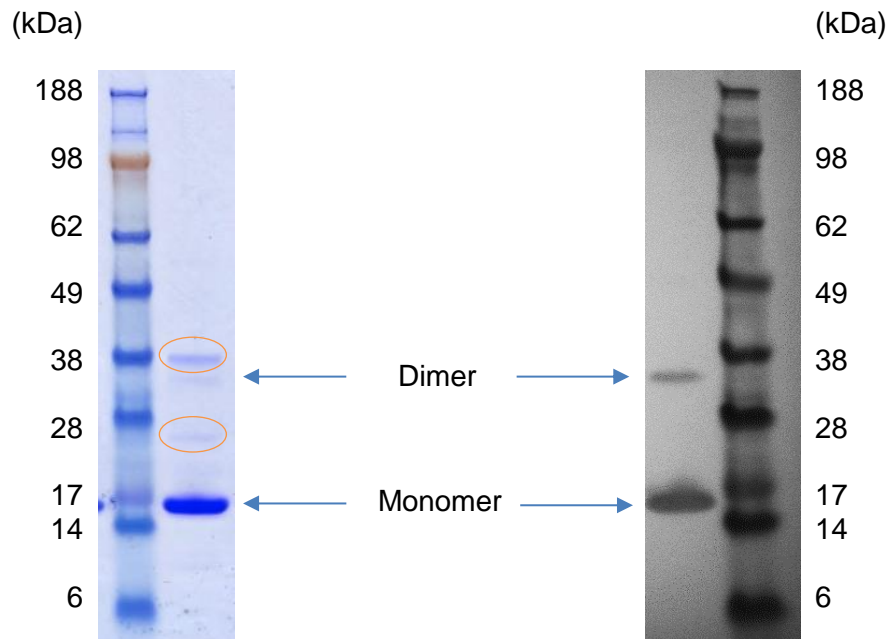
564 Biosciences, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

565 * To whom correspondence should be sent: t.w.overton@bham.ac.uk

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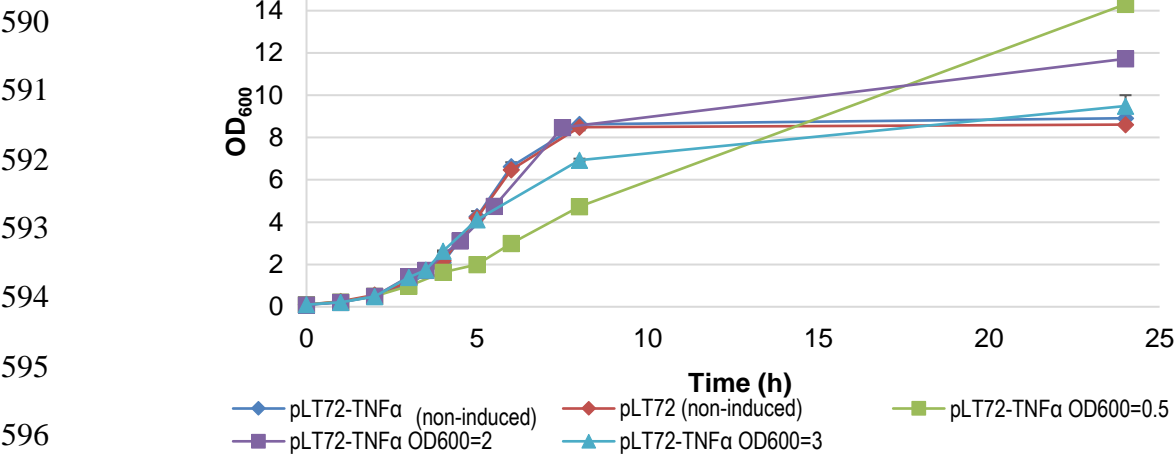
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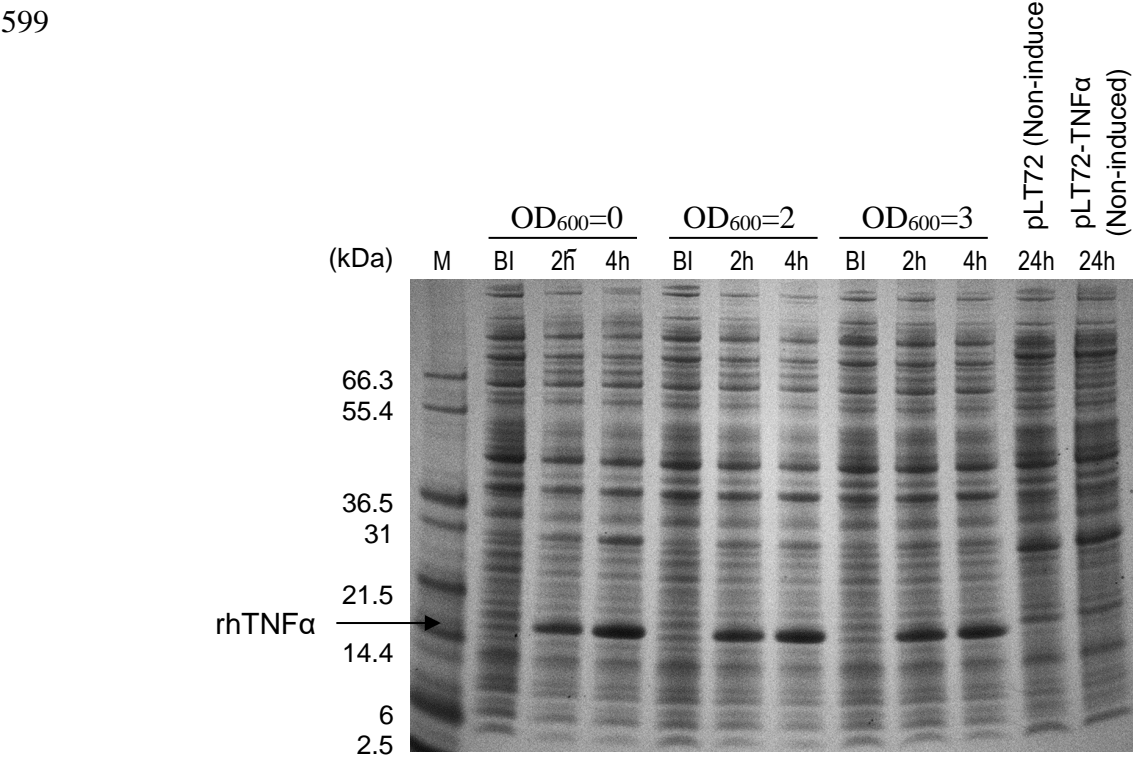
Supplemental Figure S1. Final material obtained after the purification process of rhTNF α . The rhTNF α accumulated mainly in the monomeric form. The presence of the rhTNF α dimer could also be detected by SDS-PAGE (left) and western blot (right). Bands corresponding to other contaminant proteins could also be observed (green circles). The final product had a purity greater than 95 % as determined by densitometry. The western blot was developed using an anti-TNF α antibody which confirmed the identity of the monomer, dimer and trimer forms of the rhTNF α produced by fed-batch fermentation.

588 **SUPPLEMENTAL FIGURE S2.**

589 **a)**



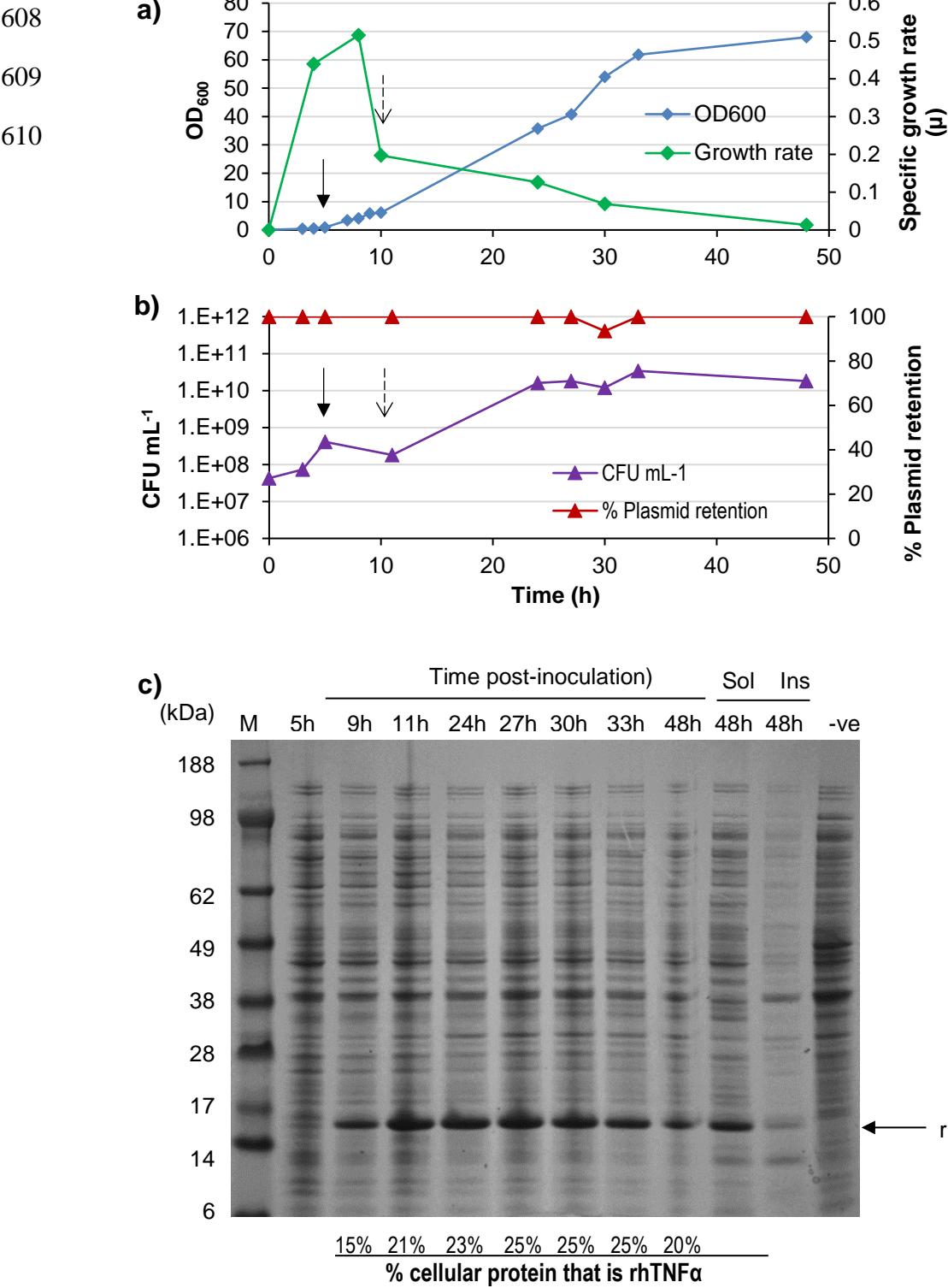
598 **b)**



600 **Supplemental Figure S2. The effect of the induction point on the production of rhTNF α .** (a)
601 Growth of *E. coli* BL21-T7 carrying the empty vector (pLT72) or the vector coding for rhTNF α (pLT72-
602 TNF α) incubated at 30 °C; cultures were induced with 0.2 % arabinose at OD₆₀₀ \approx 0.5, 2 or 3. (b)
603 SDS-PAGE showing accumulation of rhTNF α from whole cell lysates before induction (BI), 2 hours
604 and 4 hours after induction. Data shown are mean values from two replica flasks for OD₆₀₀, error
605 bars are ± 1 standard deviation.

606

607 SUPPLEMENTAL FIGURE S3



611 **Supplemental Figure S3. Production of rhTNF α by fed-batch fermentation using optimised**
612 **expression vector pLT72-T7tKan-TNF α .** *E. coli* BL21-T7 pLT72-T7tKan-TNF α was grown at 25 °C
613 in medium A and induced with 0.005 % arabinose at an OD₆₀₀ \approx 0.5 (t = 5h; solid arrow). Feeding
614 was started at t = 10h (dashed arrow). (a) Growth as determined using OD₆₀₀ and specific growth
615 rate. (b) CFU and plasmid retention. (c) SDS-PAGE analysis of whole cell lysates. The 5h sample is
616 immediately before induction. rhTNF α is indicated, along with densitometric analysis of the
617 percentage of cellular protein that is rhTNF α . The “–ve” sample is *E. coli* BL21-T7 pLT72 (empty
618 vector, non-induced) after 24 hours of growth. Samples obtained at t = 48h were also fractionated
619 into soluble (Sol) and insoluble (Ins) fractions to determine the quantities of soluble and insoluble
620 rhTNF α .

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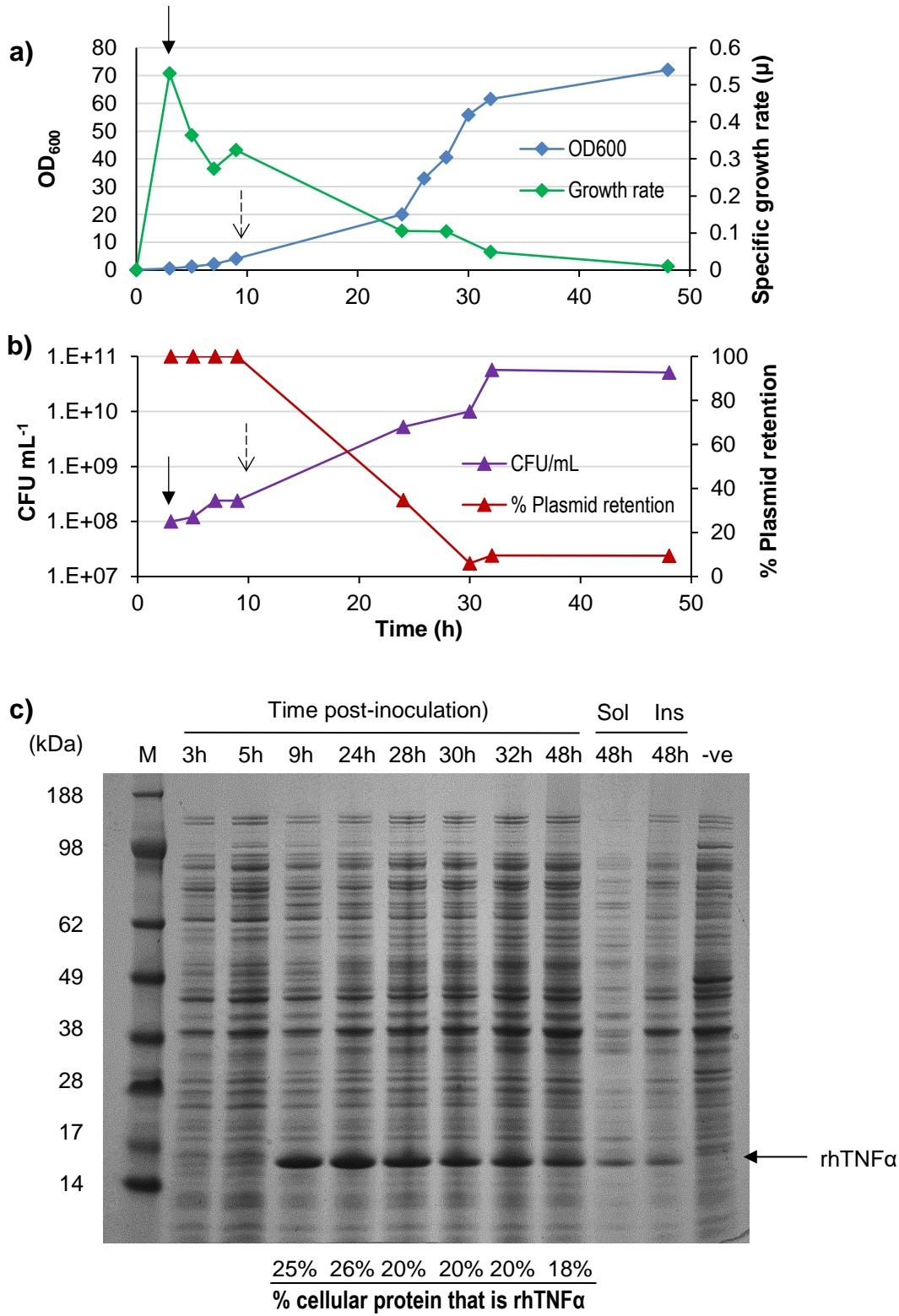
622 SUPPLEMENTAL FIGURE S4

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627 **Supplemental Figure S4. Production of rhTNF α by fed-batch fermentation without the**
628 **addition of casamino acids.** *E. coli* BL21-T7 pLT72-T7tKan-TNF α was grown in medium
629 B at 25 °C and induced with 0.005 % arabinose at an OD₆₀₀ \approx 0.5 (t = 3h; solid arrow).
630 Feeding was started at t = 10h (dashed arrow). (a) Growth as determined using OD₆₀₀ and
631 specific growth rate. (b) CFU and plasmid retention. (c) SDS-PAGE analysis of whole cell
632 lysates. The 3h sample is immediately before induction. rhTNF α is indicated, along with
633 densitometric analysis of the percentage of cellular protein that is rhTNF α . The “–ve” sample
634 is *E. coli* BL21-T7 pLT72 (empty vector, non-induced) after 24 hours of growth. Samples
635 obtained at t = 48h were also fractionated into soluble (Sol) and insoluble (Ins) fractions to
636 determine the quantities of soluble and insoluble rhTNF α .

637

638 **Supplemental Table S1.** Summary of the results obtained at the end of each of the
639 fermentation studies carried out for the production of rhTNF α .

640

| Fermentation No. | 1 | 2 | 3 | 4 |
|---|------------------------------|------------------------------|------------------------------|------------------------------|
| Figure | Fig. 5 | Fig. S3 | Fig. S4 | Fig. 7 |
| Plasmid name | pLT72-TNF α | pLT72- T7tKan-TNF α | pLT72-T7tKan-TNF α | pLT72-T7tKan-TNF α |
| Culture medium recipe | A | A | B | C |
| Final OD ₆₀₀ | 91 | 68 | 72 | 111 |
| % of cellular protein corresponding to rhTNF α | 20 % | 20 % | 18 % | 25 % |
| rhTNF α yield ^a | 48 h: 3.82 g·L ⁻¹ | 48 h: 1.92 g·L ⁻¹ | 48 h: 1.84 g·L ⁻¹ | 48 h: 5.35 g·L ⁻¹ |
| % of rhTNF α in the soluble fraction | 92 % | 80 % | 50 % | 70 % |

641

642 Footnote: ^a determined by comparison of band intensity on SDS-PAGE gels with band
643 intensity of rhTNF α standards.

644

645

646 **Supplemental methods**

647 **Fed-batch fermentation**

648 Starter cultures were grown in 10 mL of LB broth with 50 $\mu\text{g}\cdot\text{mL}^{-1}$ kanamycin at 25 °C and
649 200 rpm until $\text{OD}_{600} = 2$. Starter cultures were used to inoculate a 1 L baffled shake-flask
650 containing 200 mL of LB broth with 50 $\text{mg}\cdot\text{L}^{-1}$ kanamycin, and were grown at 25 °C and 200
651 rpm to an OD_{600} between 4 and 6.

652 A 7 L total volume (5 L working volume) bench-top fermenter (Applikon ADI 1010 Bio
653 controller) equipped with 3 Rushton impellers and 4 baffles was used for fermentation
654 experiments. The aeration rate was constant at 1 volume air per volume medium per minute
655 (vvm) and the dissolved oxygen tension (DOT) was maintained above 20 % being controlled
656 by the stirrer speed (200 - 1250 rpm). All fermentations began with an initial volume of 3 L
657 batch salts, sterilised in the vessel by autoclaving for 20 minutes at 121 °C. Once cooled,
658 post-autoclave additions and trace element solutions were added. Two litres of feed solution
659 was prepared and sterilised by filtration (0.22 μm filter). For medium A (Cobra biologics), the
660 batch salts contained: 13.3 $\text{g}\cdot\text{L}^{-1}$ K_2HPO_4 , 4 $\text{g}\cdot\text{L}^{-1}$ $(\text{NH}_4)_2\text{SO}_4$, 1.7 $\text{g}\cdot\text{L}^{-1}$ citric acid, 10 $\text{g}\cdot\text{L}^{-1}$
661 Bacto™ yeast extract and 0.16 $\text{mL}\cdot\text{L}^{-1}$ PPG 2000. The post-autoclave additions were: 1
662 $\text{mL}\cdot\text{L}^{-1}$ trace elements solution A (comprising 5 $\text{g}\cdot\text{L}^{-1}$ citric acid, 2 $\text{g}\cdot\text{L}^{-1}$ $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, 1.2 $\text{g}\cdot\text{L}^{-1}$
663 $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$, 2.5 $\text{g}\cdot\text{L}^{-1}$ H_3BO_3 , 2 $\text{g}\cdot\text{L}^{-1}$ $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$, 1.2 $\text{g}\cdot\text{L}^{-1}$ $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$), 10 $\text{mL}\cdot\text{L}^{-1}$
664 trace elements solution B (comprising 6 $\text{g}\cdot\text{L}^{-1}$ $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, 0.84 $\text{g}\cdot\text{L}^{-1}$ $\text{EDTA}\cdot 2\text{H}_2\text{O}$ and 0.8
665 $\text{g}\cdot\text{L}^{-1}$ ZnCl_2), 10 $\text{g}\cdot\text{L}^{-1}$ glycerol, 1.2 $\text{g}\cdot\text{L}^{-1}$ $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 2 % (w/v) casamino acids and 1 $\text{mL}\cdot\text{L}^{-1}$
666 50 $\text{mg}\cdot\text{mL}^{-1}$ kanamycin stock. The feed solution contained 600 $\text{g}\cdot\text{L}^{-1}$ glycerol, 5 $\text{g}\cdot\text{L}^{-1}$
667 $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 50 $\text{g}\cdot\text{L}^{-1}$ yeast extract, 10 $\text{g}\cdot\text{L}^{-1}$ KH_2PO_4 , 2.1 $\text{g}\cdot\text{L}^{-1}$ K_2HPO_4 , 2 % (w/v) casamino
668 acids, 1 $\text{mL}\cdot\text{L}^{-1}$ 50 $\text{mg}\cdot\text{mL}^{-1}$ kanamycin stock and 0.5 $\text{mL}\cdot\text{L}^{-1}$ 20 % arabinose stock. For
669 medium B, casamino acids were omitted and replaced with 14 $\text{g}\cdot\text{L}^{-1}$ $(\text{NH}_4)_2\text{SO}_4$ and 0.3 $\text{g}\cdot\text{L}^{-1}$
670 1 of $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$.

For medium C [17], the batch salts contained 14 g·L⁻¹ (NH₄)₂SO₄, 35 g·L⁻¹ glycerol, 20 g·L⁻¹ Bacto™ yeast extract, 2 g·L⁻¹ KH₂PO₄, 16.5 g·L⁻¹ K₂HPO₄, 7.5 g·L⁻¹ citric acid, 1.5 mL · L⁻¹ concentrated H₃PO₄ and 0.66 mL·L⁻¹ PPG 2000. The post-autoclave additions were 34 mL·L⁻¹ trace elements solution (comprising 3.36 g·L⁻¹ FeSO₄·7H₂O, 0.84 g·L⁻¹ ZnSO₄·7H₂O, 0.15 g·L⁻¹ MnSO₄·H₂O, 0.25 g·L⁻¹ Na₂MoO₄·2H₂O, 0.12 g·L⁻¹ CuSO₄·5H₂O, 0.36 g·L⁻¹ H₃BO₃ and 48 mL·L⁻¹ concentrated H₃PO₄), 10 mL·L⁻¹ 1 M MgSO₄·7H₂O, 2 mL·L⁻¹ 1 M CaCl₂·2H₂O and 1 mL·L⁻¹ 50 mg·mL⁻¹ kanamycin stock. The feed contained 714 g·L⁻¹ glycerol, 30 mL·L⁻¹ 1 M MgSO₄·7H₂O, 1 mL·L⁻¹ 50 mg·mL⁻¹ kanamycin stock and 0.5 mL·L⁻¹ 20 % arabinose stock.

The pH was maintained at 6.8 by the addition of 5 M NaOH and 5 M HCl for fermentations using media A and B, and at 7.0 by the addition of 5 M HCl or 20 % NH₄OH for medium C. Polypropylene glycol (PPG) antifoam was added when required. Fed-batch fermentations were monitored using BioXpert® software (Applikon). The inoculum was added to an initial OD₆₀₀ of 0.1. The fermentation was carried out at a temperature of 25 °C and the culture was induced with 0.005 % arabinose at an OD₆₀₀ of 0.5. The feed solution was started 10 h after inoculation at an exponential feed rate to achieve a specific growth rate of 0.1 h⁻¹, calculated using equation 1:

$$F = \left(\frac{1}{S}\right) \times \left(\frac{\mu}{Y_{XS}} + m\right) \times X_0 \times e^{\mu t} \quad (1)$$

F is the feed rate in L·h⁻¹, S is the substrate concentration in the feed in g·L⁻¹, μ is the required specific growth rate in h⁻¹ calculated using equation 2, Y_{XS} is the yield coefficient in g biomass per g carbon source, m is the maintenance coefficient, X_0 is the biomass in g and t is time. Values for Y_{XS} and m were obtained from the literature, 0.22 [19] and 0.025 [20], respectively.

695 **SDS-PAGE**

696 4-12 % Bis-Tris NuPAGE SDS-PAGE gels (Life Technologies) were generally used to
697 evaluate the production of recombinant proteins. Seven microliters of protein sample were
698 mixed with 2 μ L of 4x NuPAGE LDS sample buffer (Life Technologies) and 1 μ L of 10x
699 NuPAGE sample reducing agent (Life Technologies), and heated for 10 min at 70 °C. 1x
700 electrophoresis running buffer was prepared by diluting 20x NuPAGE MES SDS running
701 buffer (Life Technologies) in deionised water. For reducing protein electrophoresis, 0.5 mL
702 of NuPAGE antioxidant (Life Technologies) was added to 200 mL of running buffer and used
703 to fill the inner chamber of the electrophoresis tank. SDS-PAGE gels were run for at least
704 45 minutes at 200 V according to the manufacturers' protocol. Molecular size markers were
705 used: Mark12™ Unstained Protein Standard (Life Technologies) or SeeBlue® Plus2 Pre-
706 Stained Protein Standard (Life Technologies). SDS-PAGE gels were stained using Colloidal
707 Blue Staining (Life Technologies). SDS-PAGE gels were submerged in the fixing solution,
708 (40 % (v/v) methanol, 10 % (v/v) glacial acetic acid) for 10 minutes, the staining solution A
709 (20 % (v/v) methanol and 20 % (v/v) staining solution A) for 10 minutes, then staining solution
710 B was added to a final concentration of 5 % (v/v). SDS-PAGE gels were stained for a
711 minimum of 3 hours and de-stained with deionised water for at least 12 hours.

712 *Western blotting*

713 SDS-PAGE gels were run as above and transferred to a 0.2 μ m nitrocellulose membrane
714 (Life Technologies) using the Xcell II blot module at 30 V for 1 h (Life Technologies). Transfer
715 buffer was prepared by the addition of 20x NuPAGE transfer buffer (Life Technologies), 10
716 % of methanol (v/v) and 1 mL·L⁻¹ NuPAGE antioxidant. Membranes were blocked in 5 %
717 (w/v) skimmed milk powder (Sigma-Aldrich) in PBS for at least 1 hour. For the detection of
718 rhTNF α , the blot was incubated with an anti-TNF α antibody (ab9635, Abcam) using 1:2500
719 dilution in 5 % (w/v) skimmed milk (Sigma-Aldrich) in PBS for 1 h, washed with 0.05 %

720 Tween-20 in PBS and successively incubated with an anti-rabbit IgG antibody conjugated
721 with horseradish peroxidase (HRP; Sigma-Aldrich) using 1:3000 dilution for an hour.
722 Western blots were developed using 3,3',5,5'-tetramethylbenzidine (TMB) substrate for HRP
723 (Sigma-Aldrich).

724 *Quantification of rhTNF α from SDS-PAGE*

725 AlphaEase® software (Alpha Innotech) was used to calculate the quantity of rhTNF α as a
726 percentage of total cell protein (TCP). Gels were photographed using an Alphamager
727 (Alpha Innotech) and images subjected to background subtraction using the default settings
728 for peak-to-peak background subtraction. The percentage of soluble and insoluble
729 recombinant protein was calculated by the software package. All samples were normalised
730 by OD₆₀₀ before loading on the SDS-PAGE gel, so each lane contained equivalent biomass.

731 rhTNF α reference material obtained from Life Technologies was used to quantify the
732 concentration of rhTNF α obtained at the end of each fermentation by densitometry from
733 samples. A standard curve was generated by loading different concentrations of rhTNF α
734 reference material on an SDS-PAGE gel. The concentration of rhTNF α from fermentation
735 samples was quantified using a standard curve with the AlphaEase® software. The rhTNF α
736 yields were calculated to obtain the final yield based on the final OD₆₀₀ of the culture.

737 **Specific growth rate**

738 Specific growth rate μ was calculated according to the following equation:

739
$$\mu = \frac{\ln(X_2) - \ln(X_1)}{t_2 - t_1}$$

740 Where: X_1 and X_2 are biomass concentrations at times t_1 and t_2 .

741